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An Essential Yeast Gene Encoding a Homolog of Ubiquitin-activating Enzyme*

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Ubiquitin (Ub) activation by the Ub-activating (*E1*) enzyme is the initial and essential step common to all of the known processes that involve post-translational conjugation of Ub to itself or other proteins. The "activated" Ub, linked via a thioester bond to a specific cysteine residue of *E1* enzyme, can be transferred to a cysteine residue in one of several Ub-conjugating (*E2*) enzymes, which catalyze the formation of isopeptide bonds between the C-terminal glycine of Ub and lysine residues of acceptor proteins. In the yeast *Saccharomyces cerevisiae*, a 114-kDa *E1* enzyme is encoded by an essential gene termed *UBA1* (McGrath, J. P., Jentsch, S., and Varshavsky, A. (1991) *EMBO J.* 10, 227–236). We describe the isolation and analysis of another essential gene, termed *UBA2*, that encodes a 71-kDa protein with extensive sequence similarities to both the *UBA1*-encoded yeast *E1* and *E1* enzymes of other organisms. The regions of similarities between Uba1p and Uba2p encompass a putative ATP-binding site as well as a sequence that is highly conserved between the known *E1* enzymes and contains the active-site cysteine of *E1*. This cysteine is shown to be required for an essential function of Uba2p, suggesting that Uba2p-catalyzed reactions involve a transient thioester bond between Uba2p and either Ub or another protein. Uba2p is located largely in the nucleus. The putative nuclear localization signal of Uba2p is near its C terminus. The Uba1p (*E1* enzyme) and Uba2p cannot complement each others essential functions even if their subcellular localization is altered by mutagenesis. Uba2p appears to interact with itself and several other *S. cerevisiae* proteins with apparent molecular masses of 52, 63, 87, and 120 kDa. Uba2p is multiubiquitinated *in vivo*, suggesting that at least a fraction of Uba2p is metabolically unstable. Uba2p is likely to be a component of the Ub system that functions as either an *E2* or *E1/E2* enzyme.

Ubiquitin (Ub)[1] is a highly conserved 76-residue protein whose covalent conjugation to other proteins (often in the form of a multi-Ub chain) plays a role in a number of processes, primarily through routes that involve protein degradation (reviewed by Rechsteiner (1991), Finley and Chau (1991), Hershko and Ciechanover (1992), Jentsch (1992), Varshavsky (1992), Goldberg (1992), Hochstrasser (1992), Vierstra (1993), Parsell and Lindquist (1993), and Ciechanover (1994)). Ub activation by the Ub-activating (*E1*) enzyme is the initial and essential step common to all of the known processes that involve post-translational conjugation of Ub to itself or other proteins. The *E1* enzyme activates Ub in an ATP-dependent reaction by first adenylating the C-terminal Gly-76 of Ub and thereafter linking this residue to the side chain of a Cys residue in the same *E1* molecule, yielding an *E1*~Ub thioester and free AMP (Ciechanover *et al.*, 1982; Haas *et al.*, 1983; Pickart, 1988; Pickart *et al.*, 1994). In the presence of a Ub-conjugating (*E2*) enzyme (one of several such enzymes in a cell), the *E1*-linked Ub is transferred to a Cys residue in *E2*. The *E2* enzymes conjugate their cysteine-linked Ub moiety to its ultimate protein acceptors, yielding isopeptide bond-mediated Ub-protein conjugates (reviewed by Jentsch (1992)). The targeting of proteins for ubiquitination by at least some *E2* enzymes involves their association with distinct proteins called recognins, *E2*s or Ub-protein ligases, which mediate the initial recognition of a substrate by a recognin-*E2* complex (Dohmen *et al.*, 1991a; Sung *et al.*, 1991; Scheffner *et al.*, 1995). At least some recognins also form a thioester with Ub in the presence of *E1*, appropriate *E2*, and a "downstream" protein substrate[2] (Scheffner *et al.*, 1995), suggesting the existence of a Ub transesterification cascade (*E1* → *E2* → recognin) that mediates the formation of isopeptide bond-linked Ub-protein conjugates.

Genes encoding *E1* enzymes have been cloned from a variety of organisms, including the yeast *Saccharomyces cerevisiae*, plants, and mammals such as mouse and man (Hatfield *et al.*, 1990; McGrath *et al.*, 1991; Handley *et al.*, 1991; Imai *et al.*, 1992; Kok *et al.*, 1993; Hatfield and Vierstra, 1992). All of these genes encode 110–120-kDa proteins highly similar in sequence. *UBA1*, which encodes the 114-kDa *E1* enzyme of *S. cerevisiae*, is essential for cell viability (McGrath *et al.*, 1991). Biochemical and genetic analyses of ts85 and analogous temperature-sensitive (*ts*) mammalian cell lines demonstrated that

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) Z48725.

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[1] The abbreviations used are: Ub, ubiquitin; His6-Ub, Ub bearing a His_6-containing N-terminal extension; *E1*, Ub-activating enzyme; *E2*, Ub-conjugating enzyme; *wt*, wild-type; GFP, green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; NLS, nuclear localization signal; PCR, polymerase chain reaction; bp, base pair(s); ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis.

[2] V. Chau and A. Varshavsky, unpublished data.

these cells carry *ts* mutations in their genes for *E*1 enzyme, and that the activity of *E*1 is required for ATP-dependent proteolysis, as well as for other (possibly proteolysis-mediated) cellular functions (Finley *et al.*, 1984; Ciechanover *et al.*, 1984; Kulka *et al.*, 1988; Aysawa *et al.*, 1992; Nishitani *et al.*, 1992). A gene required for spermatogenesis was identified in the *Sxr* (sex-reversed) region of the mouse Y chromosome and shown to encode an *E*1 enzyme whose expression is testis-specific (Mitchel *et al.*, 1991; Kay *et al.*, 1991).

We report the isolation and analysis of a novel essential *S. cerevisiae* gene, termed *UBA2*, that encodes a 636-residue protein whose amino acid sequence is highly similar to those of *E*1 enzymes, but whose activity appears to be distinct from that of the known *E*1 enzymes.

MATERIALS AND METHODS

Media, Genetic Techniques, and Construction of Strains—*S. cerevisiae* cultures were grown at 30 °C in rich (YP) or synthetic (S) media (Sherman *et al.*, 1986) containing either 2% dextrose (YPD or SD media) or 2% galactose (YPG or SG media). Transformation of *S. cerevisiae* was carried out as described by Dohmen *et al.* (1991b). The strain SUB312 (a gift from Dr. D. Finley) (*MATa ura3–52 ubi1Δ::TRP1 ubi2-Δ2::ura3 ubi3-Δub2 ubi4-Δ2::*LEU2 (pUB100, expressing a non-Ub portion of Ubi1p) (pUB23-X, expressing Ub-βgal from P_{GAL1}) lacks chromosomal sequences encoding Ub and expresses Ub (an essential protein) from the P_{GAL1} promoter in the plasmid pUB23 (Finley *et al.*, 1994). In the present work, pUb23 of SUB312 were replaced with plasmids YEp96 or YEp105, which expressed Ub or mycUb, respectively, from the P_{CUP1} promoter (Ellison and Hochstrasser, 1991). All other *S. cerevisiae* strains were derivatives of JD47-13C (*MATa leu2–3, 112 lys2–801 his3-Δ200 trp1-Δ63 ura3–52*) (Madura *et al.*, 1993). JD51, a diploid derivative of JD47-13C, was constructed through a transient expression of HO endonuclease from the plasmid YCp50-HO (Herskowitz and Jensen, 1986).

The diploid JD90 (*uba2Δ::HIS3/UBA2*) was produced from JD51 using one-step gene transplacement (Rothstein, 1991). The *Pst*I DNA fragment containing the *uba2Δ::HIS3* allele used in the transplacement was isolated from pJD346. The latter plasmid was constructed using oligonucleotide-directed deletion (Finley *et al.*, 1987) of the *UBA2* gene and simultaneous introduction of a *Bam*HI site, which was used to insert the *HIS3* marker as an 1.8-kilobase pair *Bam*HI fragment (*HIS3* was isolated from YEp6; Struhl *et al.* (1979)). The strain JD62 (P_{GAL1}-*UBA2/UBA2*) was produced by integrating the plasmid pJDA9-5 (which had been linearized by a cut with *Bst*XI in the *UBA2* part of the plasmid) into the *UBA2* locus of the strain JD51. pJDA9-5 was constructed in the background of the vector pRS306 (Sikorski and Hieter, 1989) and contained 470 bp of *UBA2*, from the *Eco*RI site (which was introduced immediately in front of the ATG start codon using polymerase chain reaction (PCR)) to the *Sma*I site. This 470-bp fragment was ligated to a 500-bp fragment containing the P_{GAL1} promoter. The latter fragment was generated by first introducing, using oligonucleotide-directed mutagenesis, a *Sma*I site between the promoters P_{GAL1} and P_{GAL10} in a fragment originally isolated from of pBM272 (Hovland *et al.*, 1989), and thereafter cloning the resulting *Sma*I-*Bam*HI fragment into the polylinker of pRS306. Integration of the resulting (linearized) pJDA9-5 at the *UBA2* locus of the diploid strain JD51 yielded, in one of the two *UBA2*-containing chromosomes of JD51, a nonfunctional (truncated) copy of *UBA2*, and the intact *UBA2* open reading frame (ORF) expressed from P_{GAL1}. JD62-6A is a haploid ***MAT*a** segregant of the strain JD62 bearing the altered *UBA2* locus.

JD77 is a *uba1Δ::HIS3/UBA1* derivative of strain JD51. The *uba1Δ::HIS3* allele was produced as described by McGrath *et al.* (1991). JD77 was transformed with pJD320, a derivative of the *CEN6/ARSH4/URA3* vector pRS316 (Sikorski and Hieter, 1989) that expressed *UBA1* from the P_{GAL1} promoter. The P_{GAL1}-*UBA1* portion of pJD320 was constructed by placing, using PCR, an *Eco*RI site immediately upstream of the ATG start codon of *UBA2*. JD77-1A is a haploid ***MAT*a** segregant of strain JD77 that carries pJD320.

Cloning of UBA2 and Construction of Plasmids—Standard methods were used (Ausubel *et al.*, 1992). The *UBA2* gene was initially identified in a library of λgt11 phages (Young and Davis, 1983) carrying inserts of *S. cerevisiae* genomic DNA. Specifically, *UBA2* was isolated as a result of a cross-reaction of a *UBA2*-expressing phage plaque with a polyclonal antiserum raised against a yeast plasma membrane ATPase kinase.[3] An oligonucleotide constructed on the basis of initial sequence analysis was then used to isolate *UBA2* clones through plaque hybridization with the EMBL3A phage library of *S. cerevisiae* genomic DNA (a gift from Dr. R. Young). For mapping and sequencing of *UBA2*, yeast DNA fragments derived from two overlapping EMBL3A phage inserts were subcloned into YCplac22 (Gietz and Sugino, 1988), and a restriction map of *UBA2* was produced using standard methods. The sequence of *UBA2* was determined on both strands using a variety of *UBA2* subclones, synthetic oligonucleotides as primers, the chain termination method (Ausubel *et al.*, 1989), and a Sequenase Kit (U. S. Biochemical Corp.).

A ~3-kilobase pair *Pst*I fragment of the YCplac22-based *UBA2*-bearing plasmid pJDA2-A contained the *UBA2* ORF, ~an 0.6 kilobase pair of the 5′-flanking sequence, and a 3′-flanking region of the *UBA2* locus that included part of the adjacent *SAC7* gene (Dunn and Shortle, 1990). The *Pst*I fragment was subcloned into the phagemid pRS315 (Sikorski and Hieter, 1989), yielding pJDA315. (One *Pst*I site of this fragment was present in the polylinker of YCplac22; the other *Pst*I site was present 3′ to the insert/EMBL3A junction.) In the construction of the *uba2Δ*, *uba2-C177A*, and *uba2-C177S* alleles of *UBA2* (see the main text), single-stranded pJDA315 DNA and synthetic oligonucleotides were used as described by Kunkel (1985). To express *UBA2* from P_{CUP1} (derived from YEp46; see below) or P_{GAL1} (see above), a *Bgl*II site was placed in front of the ATG start codon of *UBA2* using PCR and synthetic primers.

For C-terminal tagging of Uba2p, a *Kpn*I recognition sequence was linked to the 3′ end of the *UBA2* ORF using PCR. This *Kpn*I site was then used to link, in-frame, the *UBA2* ORF and fragments encoding specific sequence tags. A restriction fragment encoding 23 residues and containing two repeats of the 9-residue ha epitope (Field *et al.*, 1988) was a gift from Dr. N. Schnell. The FLAG tag (Brizzard *et al.*, 1994) and the His6 tag (Hoffmann and Roeder, 1991) were produced using PCR and synthetic primers. The GFP gene (Chalfie *et al.*, 1994) was amplified from the plasmid TU#65 (a gift from Dr. N. Johnsson) using PCR and primers that added appropriate restriction sites for in-frame fusions with *UBA2*-derived genes (see above). In the constructs containing untagged *UBA2*, this gene was flanked by its original 3′ sequences, whereas the tagged *UBA2* derivatives bore T_{CYC1}, the transcription termination sequence of the *CYC1* gene that was transplanted from the plasmid YEp46 (Ecker *et al.*, 1987) (a gift from Dr. D. Finley). The tagged derivatives of *UBA1* and the chimeric genes produced by swapping sequences of *UBA1* and *UBA2* were constructed using PCR-generated fragments of these genes.

The final constructs were inserted into the 2μ-based plasmids YEplac181 (*LEU2*) or YEplac195 (*URA3*) (Gietz and Sugino, 1988), using multistep protocols (details available on request). For the expression of His6-Ub (Ub bearing a Met-Arg-Gly-Ser-His_6-Gly-Ser N-terminal extension) in *Escherichia coli*, a DNA fragment encoding the Ub ORF was amplified from plasmid YEp46 using PCR and synthetic primers, and cloned as a *Bam*HI-*Kpn*I fragment into pQE-40 (Qiagen), yielding pJD359. To express a His6-tagged Ub (Beers and Callis, 1993) in *S. cerevisiae*, an *Eco*RI-*Kpn*I fragment from pJD359 encoding His6-Ub was inserted between P_{CUP1} and T_{CYC1} (both from YEp46) in the background of YEplac181 (see above), yielding pJD421. Most of the junctures and tags generated by PCR and synthetic oligonucleotides were verified by sequencing. In the cases where sequence testing was incomplete, biological functions of the constructs were verified using independently produced clones.

Immunoprecipitation of Radiolabeled Proteins—The *S. cerevisiae* strain JD62-6A was transformed with either YEplac181 (a vector), pJD389, pJD302, pJD306, or pJD396 that expressed, respectively, no Uba2p, Uba2p-ha, Uba2p-C177A-ha, Uba2p-C177S-ha, or Uba2p-GFP-ha. The cultures were grown in SG medium to A_{600} of ~1, then diluted 5-fold into SD or SG media containing 50 μM $CuSO_4$, and incubated for 8 h. The cells from 15-ml cultures were collected by centrifugation, resuspended in 0.6 ml of SD or SG media containing 50 μM $CuSO_4$ and 0.1 mCi of Tran^{35}S-label (ICN), and incubated for 10 min at 30 °C. The cells were collected by centrifugation for 10 s at 14,000 × *g* and resuspended in 600 μl of the lysis buffer (0.15 M NaCl, 1% Triton X-100, 1 mM EDTA, 50 mM Na-HEPES, pH 7.5) containing also 10 mM *N*-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, as well as leupeptin, pepstatin A, antipain, chymostatin, and aprotinin (each at 20 μg/ml). 0.5 ml of 0.5-mm glass beads was then added, and the cells were lysed by vortexing in the lysis buffer. The extracts were centrifuged at 14,000 × *g* for 15 min. Immunoprecipitations were carried out with the supernatants (containing approximately equal amounts of $CHCl_3COOH$-pre-

[3] H. Forrová, J. Kolarov, and A. Goffeau, unpublished data.

cipitable ^{35}S) by adding ascitic fluid (1.5 μl/0.6 ml of supernatant) containing the monoclonal anti-ha antibody 12CA5 (Field *et al.*, 1988) (Babco Inc., Richmond, CA). The samples were incubated on ice for 2 h; 25 μl of Protein A-Sepharose (0.1 g/ml) (Pharmacia Biotech Inc.) was then added, and the suspensions were incubated, with end-over-end rotation, for 1 h at 4 °C, followed by a 5-s centrifugation in a microcentrifuge. Pellets were washed four times with 1 ml of the lysis buffer, resuspended in the thioester-preserving electrophoretic sample buffer (10% glycerol, 2% SDS, 0.1% bromphenol blue, 50 mM Tris-HCl, pH 6.8), and centrifuged at 14,000 × *g* for ~20 s. One-half of each supernatant was left untreated; another half was made 0.25 M in β-mercaptoethanol, followed by incubation at 100 °C for 3 min. Both samples were subjected to SDS-8% PAGE and fluorography. ^{14}C-Labeled protein size standards were from Amersham.

Immunoblot Analysis of Immunoprecipitated Proteins—The strain SUB312 (Finley *et al.*, 1994), which expressed either wild-type (*wt*) Ub or mycUb from plasmids YEp96 or YEp105, respectively (see above), was transformed with either the vector YEplac195 (Gietz and Sugino, 1988), its derivative pJD426, or its derivative pJD428, which expressed, respectively, Uba1p-FLAG or Uba2p-FLAG. Cells from exponential cultures (A_{600} of ~1) of the transformants growing in SD media containing 50 μM $CuSO_4$ were collected by centrifugation, and extracts were prepared as described above. Total protein concentration in an extract was determined using the Bradford assay (Bio-Rad). An extract containing 50 μg of total protein was added to 40 μl of an agarose affinity gel containing the monoclonal anti-FLAG (M2) antibody (Eastman Kodak), followed by incubation for 1 h at 4 °C, with end-over-end rotation. The anti-FLAG resin was then washed four times with 1 ml of the lysis buffer, and the retained proteins were fractionated by SDS-8% PAGE, followed by electroblotting onto nitrocellulose membranes (Schleicher & Schuell). Sequential detection of immunoreactive proteins with the anti-FLAG or anti-myc antibodies was carried out using the Amersham ECL detection system.

Detection of Ub Thioesters—*S. cerevisiae* SUB312 cells carrying YEp96 (which expressed *wt* Ub; see above) and either pJD426 or pJD428 (which expressed, respectively, Uba1p-FLAG or Uba2p-FLAG from P_{CUP1}) were grown in SD media. The expression of FLAG-tagged proteins was induced with 50 μM $CuSO_4$ for 6 h before making the extracts, which were prepared as described above except that *N*-ethylmaleimide was replaced with 0.5 mM dithiothreitol. The FLAG-tagged proteins were immunoprecipitated as described above from extracts containing 50 μg of total protein. The anti-FLAG resin was washed twice with 1 ml of the lysis buffer and once with 1 ml of 10 mM $MgCl_2$, 25 mM Tris-HCl (pH 8.0) (Tris-Mg buffer). The washed resin was resuspended in 50 μl of the thioester assay buffer (10 mM $MgCl_2$, 1 mM ATP, 10 mM creatine phosphate, 25 mM Tris-HCl (pH 8.0)) containing also, 100 μg/ml creatine kinase and ~30 pmol of [^{35}S]methionine-labeled His6-Ub (10^6 cpm) (see below), and incubated at 37 °C for 10 min. The resin was then washed twice with Tris-Mg buffer, and resuspended in electrophoretic sample buffer (see above) containing or lacking 0.25 M β-mercaptoethanol. The former (but not the latter) sample was incubated at 100 °C for 3 min before SDS-8% PAGE. The gel was stained with Coomassie (to verify the presence of approximately equal amounts of FLAG-tagged test proteins), then dried and subjected to autoradiography.

Ub Affinity Chromatography—The strain SUB312, lacking pUB23 (see above) and expressing His6-Ub from pJD421, was transformed with plasmids pJD426 or pJD428, which expressed, respectively, Uba1p-FLAG or Uba2p-FLAG. Cells grown in 1 liter of SD, 50 μM $CuSO_4$ medium to A_{600} of ~1 were lysed with glass beads in 20 ml of 1% Triton X-100, 0.3 M NaCl, 0.33 mM β-mercaptoethanol, 50 mM sodium phosphate (pH 8.0). The extracts (diluted 4-fold) were adjusted to 0.25% Triton X-100, 1 M NaCl and loaded onto Ni-NTA-Sepharose columns (Qiagen) that bind the His6 tag. The columns were washed with 0.3 M NaCl, 5 mM $MgCl_2$, 0.33 mM β-mercaptoethanol, 10 mM imidazole, 2 mM ATP, 50 mM sodium phosphate (pH 8.0). The Uba1p-FLAG protein was eluted with the same buffer containing 2 mM AMP and 40 μM sodium pyrophosphate instead of ATP. The His6-Ub bound to the resin was subsequently eluted with a low pH buffer (Beers and Callis, 1993). Fractions were assayed for the presence of FLAG-tagged proteins by immunoblotting with the anti-FLAG antibody.

Purification of Uba2p-FLAG-His6—Strain SUB312 carrying plasmids YEp105 and pJD427, and expressing, respectively, mycUb and Uba2p-FLAG-His6, were grown in 1 liter of SD, 0.1 mM $CuSO_4$ medium to A_{600} of ~0.8. The cells were washed with 0.3 M NaCl, 50 mM sodium phosphate (pH 8.0), and lysed with glass beads in 10 ml of 5% glycerol, 1% Triton X-100, 0.3 M NaCl, 5 mM $MgCl_2$, 10 mM *N*-ethylmaleimide, 50 mM sodium phosphate (pH 8.0), and a set of protease inhibitors as described above. The extracts were centrifuged at 14,000 × *g* for 15 min. The supernatant was adjusted to a final concentration of 1 M NaCl, 1 mM imidazole, 0.5 mM β-mercaptoethanol in a final volume of 30 ml, and loaded onto a Ni-NTA-Sepharose column (at a flow rate of 0.2 ml/min). The column was washed with 30 ml of pH 8 buffer (5% glycerol, 5 mM $MgCl_2$, 1 mM imidazole, 1 M NaCl, 0.5 mM β-mercaptoethanol, 50 mM sodium phosphate (pH 8.0)), and thereafter with 45 ml of pH 6 buffer (the same as pH 8 buffer but containing 10 mM imidazole and adjusted to pH 6.0 with 1 M NaOH). Uba2p-FLAG-His6 was eluted with pH 3 buffer (the same as pH 8 buffer but adjusted to pH 3 with 1 M HCl). The eluted fractions (12 ml) were adjusted (in a final volume of 25 ml) to 8 M urea, 0.1 M sodium phosphate, 10 mM Tris (pH 8.0), and loaded onto a second Ni-NTA-Sepharose column that had been equilibrated with the same pH 8-urea buffer. The column was washed with 25 ml of pH-urea buffer followed by elution of Uba2p-FLAG-His6 with pH 6-urea buffer (the same as pH 8-urea buffer but adjusted to pH 6 with 1 M HCl). Proteins in the eluted fractions were assayed for the presence of Uba2p-FLAG-His6 and mycUb by immunoblotting, using the M2 anti-FLAG and 9E10 anti-myc antibodies, respectively.

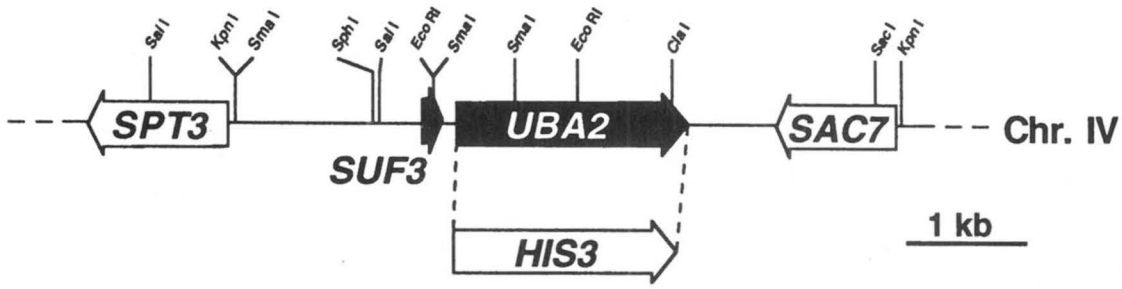
Preparation of in Vivo Labeled His6-Ub from E. coli—*E. coli* JM105 (Ausubel *et al.*, 1989), transformed with the plasmid pJD395 that expressed His6-Ub (see above), were grown in 20 ml of Luria broth containing 50 μg/ml ampicilin to A_{600} of ~0.6. The cells were centrifuged at 5,000 × *g* for 10 min, washed in M9 medium (Ausubel *et al.*, 1989), and resuspended in M9 medium supplemented with glucose (0.2%), thiamine (2 μg/ml), methionine assay medium (0.06%) (Difco), and isopropylthiogalactoside (1 mM). The cells were incubated in this medium at 37 °C for 1 h. 0.3 mCi of [^{35}S]Prolabel (Amersham) was then added, followed by incubation at 37 °C for 30 min. The cells were collected by centrifugation at 5,000 × *g* for 10 min, washed once in 0.3 M NaCl, 50 mM sodium phosphate (pH 8.0), and resuspended in 0.4 ml of the same buffer containing 5 mM β-mercaptoethanol and protease inhibitors (see above). The cells were lysed by vortexing with 0.1-mm glass beads for 1 min three times. The extracts were centrifuged at 14,000 × *g* for 15 min. [^{35}S]Methionine-labeled His6-Ub was purified from the supernatants using Ni-NTA-Sepharose (Qiagen) and the manufacturer's recommendations.

RESULTS AND DISCUSSION

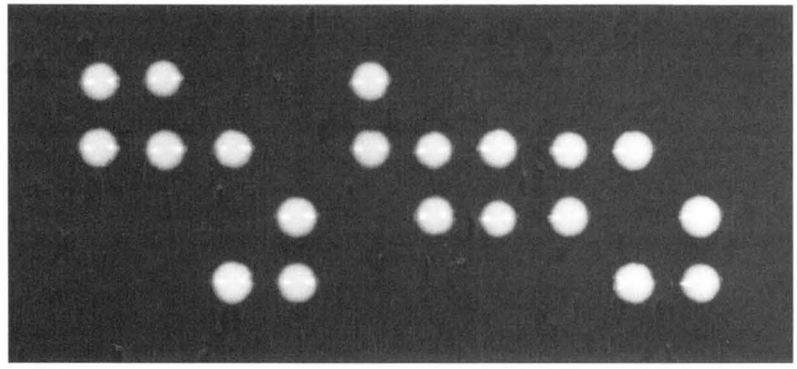
The UBA2 Gene—A DNA fragment containing a *S. cerevisiae* gene that was later termed *UBA2* was isolated from a library of λgt11 phages (Young and Davis, 1983) carrying inserts of *S. cerevisiae* genomic DNA. The finding of *UBA2* resulted from a cross-reaction of a *UBA2*-expressing phage plaque with a polyclonal antiserum raised against a yeast plasma membrane ATPase kinase, a presumably unrelated protein.[3] Initial analyses of a partial ORF in a cloned DNA fragment revealed extensive similarities of the deduced amino acid sequence to the sequences of Ub-activating (*E*1) enzymes (see below). A *S. cerevisiae* DNA clone containing the complete gene (termed *UBA2*) and its flanking sequences was then isolated (see "Materials and Methods"). Mapping and sequence analyses showed that *UBA2* is located on the right arm of Chromosome IV, flanked by *SAC7* and *SUF3* (Fig. 1*A*). *SUF3* encodes a glycine $tRNA_{CCC}$. The 3′-end of this gene is only 161 bp away from the presumed *UBA2* start codon, inferred so as to yield the largest ORF. The next in-frame ATG is located 126 bp downstream of the inferred start codon. A protein initiated from this downstream ATG would lack a putative ATP-binding motif (see below).

The 1908-bp *UBA2* encodes an acidic (calculated pI of 4.8), 636-residue (71 kDa) protein (Fig. 1*C*). The Codon Adaptation Index (calculated according to Sharp and Li (1987)) of *UBA2* is 0.151, characteristic of weakly expressed yeast genes. The sequence of the first 561 residues of Uba2p is highly similar (28% identical, 49% similar) to the sequence between residues 414 and 1020 of the 114-kDa Uba1p, the yeast *E*1 enzyme (Fig. 2). The sequence of Uba2p is also highly similar to those of human, mouse, and wheat *E*1 enzymes (Fig. 2). By contrast, the 636-residue Uba2p is only 12% identical (and lacks regions of contiguous identity longer than 3 residues) to the 540-residue Axr1p protein of *Arabidopsis thaliana* that is required for auxin response in this plant; the sequence of Axr1p has signif-

A



B



C

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 121 TATTTAATGATATATCATAAGGACGGTAATAAAGTCTTAAAAGAGTAGTTTTTGAGCTTTTTCTTTCCCCCCTTCAAGTAATTATTTAGAATAAAAAGTAGTGTTTAAAGACGACATTTT
 241 GGAGCATGCACTGTATAACCAGGTTCAAAAAAATTTCGCCTGCTATTGTTTTCTTTATTTTGAAGATGTGAGAAAAGTCTCATATCTAGTGAGCTTCAGTCTGTTTTTAGATGTAGTTAG
 361 CAATTACTATTTTAGGTATTTCTTTGCGCGGTTACGGTCTCAAGAATTTATATTACCACAAAACCTATGTAAGCTAGATCTTCTTAGCGAAAGTGGTTCAGTGGTTAGAATTCATGCTTC
 481 CCAAGCATGGGGCCCGGGTTCGATTCCCGGCTTCCGCATTTTTTGTGCAATTTTTTTTTATACTCTATTTTCAAGTTAAAGTTTTTCAAAGGCGAAGTATGTTTACGCTAAATCAAGATA
 601 TTTGGATGAGCAATCCACTAATTGCGTACTTCAACAGACACACAAGATCAGGAGCGCTACGCCAAACAAGAAAAGAAAATGCCAAGGGAAACAAGTTTGGTAACAATCATCGGTGAAGAT
                                                                                                  M  P  R  E  T  S  L  V  T  I  I  G  E  D    14
 721 AGTTACAAGAAATTACGGTCCTCAAGATGTCTTCTGGTCGGTGCTGGGGGCATAGGGTCTGAATTGTTAAAGGATATAATCTTGATGGAATTTGGTGAAATACATATTGTCGATTTGGAC
       S  Y  K  K  L  R  S  S  R  C  L  L  V  G  A  G  G  I  G  S  E  L  L  K  D  I  I  L  M  E  F  G  E  I  H  I  V  D  L  D    54
 841 ACCATTGACCTTTCGAACTTAAACAGGCAATTTCTTTTTAGACAAAAAGATATTAAACAACCGAAGTCTACCACAGCCGTAAAAGCAGTACAGCATTTCAATAACTCTAAGTTAGTGCCT
       T  I  D  L  S  N  L  N  R  Q  F  L  F  R  Q  K  D  I  K  Q  P  K  S  T  T  A  V  K  A  V  Q  H  F  N  N  S  K  L  V  P    94
 961 TACCAAGGAAATGTTATGGACATTTCTACCTTCCCACTGCATTGGTTTGAGCAATTTGACATTATCTTCAATGCTCTTGATAACTTGGCCGCAAGACGGTATGTCAATAAAATATCACAG
       Y  Q  G  N  V  M  D  I  S  T  F  P  L  H  W  F  E  Q  F  D  I  I  F  N  A  L  D  N  L  A  A  R  R  Y  V  N  K  I  S  Q   134
1081 TTTCTATCACTGCCCTTAATAGAATCTGGAACAGCTGGATTTGATGGATACATGCAGCCTATTATCCCCGGGAAAACTGAGTGTTTTGAATGTACAAAGAAGGAAACACCAAAGACTTTT
       F  L  S  L  P  L  I  E  S  G  T  A  G  F  D  G  Y  M  Q  P  I  I  P  G  K  T  E  C  F  E  C  T  K  K  E  T  P  K  T  F   174
1201 CCTGTGTGTACTATCAGGTCCACTCCTTCTCAGCCAATTCATTGCATTGTTTGGGCGAAAAACTTTCTATTTAACCAGCTATTTGCGTCAGAGACCTCTGGAAATGAAGATGATAACAAC
       P  V  C  T  I  R  S  T  P  S  Q  P  I  H  C  I  V  W  A  K  N  F  L  F  N  Q  L  F  A  S  E  T  S  G  N  E  D  D  N  N   214
1321 CAGGATTGGGGTACAGATGATGCTGAAGAAATTAAGCGCATTAAACAAGAAACTAATGAATTGTATGAATTGCAAAAAATTATCATATCAAGAGATGCATCCCGTATTCCAGAGATTCTT
       Q  D  W  G  T  D  D  A  E  E  I  K  R  I  K  Q  E  T  N  E  L  Y  E  L  Q  K  I  I  I  S  R  D  A  S  R  I  P  E  I  L   254
1441 AATAAATTATTCATTCAGGATATAAATAAACTGCTAGCCATTGAAAATTTATGGAAAACGAGAACAAAGCCAGTACCATTATCAGACTCTCAAATAAATACTCCTACTAAAACAGCTCAG
       N  K  L  F  I  Q  D  I  N  K  L  L  A  I  E  N  L  W  K  T  R  T  K  P  V  P  L  S  D  S  Q  I  N  T  P  T  K  T  A  Q   294
1561 TCTGCCTCTAATTCTGTTGGCACAATACAGGAGCAAATTAGTAATTTCATTAATATTACACAAAAATTAATGGATCGCTATCCCAAAGAGCAAAATCATATTGAATTTGATAAAGATGAT
       S  A  S  N  S  V  G  T  I  Q  E  Q  I  S  N  F  I  N  I  T  Q  K  L  M  D  R  Y  P  K  E  Q  N  H  I  E  F  D  K  D  D   334
1681 GCTGATACTTTGGAATTCGTCGCAACTGCTGCGAACATAAGGTCACACATATTCAATATTCCAATGAAGTCCGTATTTGACATCAAGCAAATTGCTGGTAACATTATCCCGGCTATTGCA
       A  D  T  L  E  F  V  A  T  A  A  N  I  R  S  H  I  F  N  I  P  M  K  S  V  F  D  I  K  Q  I  A  G  N  I  I  P  A  I  A   374
1801 ACAACGAATGCTATCGTTGCCGGTGCATCGTCATTGATTTCACTGCGCGTTTTAAATCTTTTGAAATATGCCCCAACTACTAAGTATACTGATTTGAATATGGCTTTCACAGCAAAGGCA
       T  T  N  A  I  V  A  G  A  S  S  L  I  S  L  R  V  L  N  L  L  K  Y  A  P  T  T  K  Y  T  D  L  N  M  A  F  T  A  K  A   414
1921 AGCAATCTATCCCAAAATCGTTATTTATCAAATCCGAAACTAGCCCCTCCAAATAAAAATTGCCCAGTTTGCTCTAAAGTTTGTAGAGGTGTTATTAAGTTATCCAGCGATTGTTTGAAC
       S  N  L  S  Q  N  R  Y  L  S  N  P  K  L  A  P  P  N  K  N  C  P  V  C  S  K  V  C  R  G  V  I  K  L  S  S  D  C  L  N   454
2041 AAAATGAAACTAAGTGATTTTGTAGTTTTAATACGCGAGAAGTACTCATATCCACAGGATATTTCGCTCCTGGACGCAAGTAATCAAAGGTTACTATTTGATTACGATTTCGAGGATTTG
       K  M  K  L  S  D  F  V  V  L  I  R  E  K  Y  S  Y  P  Q  D  I  S  L  L  D  A  S  N  Q  R  L  L  F  D  Y  D  F  E  D  L   494
2161 AACGATCGTACTTTATCGGAGATTAATCTTGGAAATGGTTCTATAATATTATTCTCCGACGAAGAAGGTGATACAATGATCCGCAAAGCCATAGAATTATTTCTTGATGTTGATGATGAA
       N  D  R  T  L  S  E  I  N  L  G  N  G  S  I  I  L  F  S  D  E  E  G  D  T  M  I  R  K  A  I  E  L  F  L  D  V  D  D  E   534
2281 CTCCCATGTAATACTTGCAGCTTACCGGACGTTGAAGTCCCATTAATAAAGGCCAACAATAGTCCTTCAAAAAATGAAGAAGAAGAAAAAAATGAAAAGGGTGCAGATGTAGTCGCAACT
       L  P  C  N  T  C  S  L  P  D  V  E  V  P  L  I  K  A  N  N  S  P  S  K  N  E  E  E  E  K  N  E  K  G  A  D  V  V  A  T   574
2401 ACGAACAGTCATGGGAAAGATGGTATCGTGATATTAGACGACGATGAAGGTGAGATCACCATCGATGCTGAACCGATCAATGGTTCAAAGAAGAGGCCAGTTGATACTGAGATTTCTGAG
       T  N  S  H  G  K  D  G  I  V  I  L  D  D  D  E  G  E  I  T  I  D  A  E  P  I  N  G  S  K  K  R  P  V  D  T  E  I  S  E   614
2521 GCGCCCAGTAACAAAAGGACAAAGTTAGTTAATGAACCGACTAATTCTGATATTGTTGAATTAGACTAATAAATAAGAAAAGGTATCTATATATTTATTTAATCGTGGTTCGCAGTGTAC
       A  P  S  N  K  R  T  K  L  V  N  E  P  T  N  S  D  I  V  E  L  D  *  *                                                  636
2641 GACATAAATGTTGCAGAGAAAATGCTTAATGATCATGATCCCTGTTATAGCTTATATGAAACAGCCAGTCATATAACATAGAAATTCATTATTGAAGAGTTACAAGCTGTAACATTTCTC
2761 TGGCATATAACAA
```

FIG. 1.

icant similarities to sequences of the known *E*1 enzymes (Fig. 2). Although both *E*1 and *E*2 enzymes interact with Ub and form enzyme ~ Ub thioesters, there are no statistically significant sequence similarities between the known *E*1s and *E*2s (Jentsch, 1992). Sequence comparisons failed to detect significant similarities between Uba2p and the known *E*2 enzymes.

The sequence similarities between Uba2p and *E*1 enzymes include the consensus sequence for a nucleotide binding site, G*X*G*XX*G (positions 28–33 in Uba2p) (Wierenga and Hol, 1983), and also the consensus sequence K*XX*PZCT*XXXX*P (Z is a non-polar residue) (positions 172–183 in Uba2p) (Fig. 2) around the essential cysteine (C) in *E*1 enzymes that becomes linked to Ub in an *E*1~Ub thioester (Hatfield and Vierstra, 1992). These motifs are present in all of the known *E*1 enzymes, but are absent from the Axr1p protein (Fig. 2). The known *E*1 enzymes contain an N-terminal domain of ~400 residues that are absent from Uba2p (Fig. 2). Conversely, the 82-residue C-terminal region of Uba2p is absent from the known *E*1 enzymes. This region contains the sequence KRTK (positions 619–622) that matches one consensus sequence for nuclear localization signals (NLSs).

The known *E*1 enzymes contain two ~150-residue regions of similar sequence (Fig. 2). Only one such region is present in Uba2p (positions 12–156); it contains stretches of similarity to the N-terminal region of the *E. coli* ClnNp protein, which functions in the biosynthesis of the organic component of molybdopterin, a molybdenum-containing prosthetic cofactor (McGrath *et al.*, 1991; Johnson and Rajagopalan, 1987; and data not shown). (The ~150-residue duplication in the *E*1 enzymes are the reason for a difference between their alignment with the *A. thaliana* Axr1p in Fig. 2 and the alignment described by Leyser *et al.* (1993).)

UBA2 Is Essential for Cell Viability—A null allele of *UBA2* was produced by replacing the entire *UBA2* ORF with the *HIS3* gene (Fig. 1*A*). The resulting construct was used to replace, by homologous recombination (Rothstein, 1991), one of the two copies of *wt UBA2* in the diploid JD51. The resulting *uba2*Δ/*UBA2* strain was sporulated and subjected to tetrad analysis. In virtually all of the 20 tetrads examined, no more than 2 of the 4 spores gave rise to growing colonies (Fig. 1*B*). All of the viable spores were His^-, indicating that the absence of *UBA2* is incompatible with spore viability. Microscopic examinations of germinated *uba2*Δ spores showed growth-arrested microcolonies of 50–100 enlarged cells. Microdissection of such colonies yielded ~60% cells with large buds, ~10% with two buds, and ~30% without buds (data not shown).

To determine whether *UBA2* is also required for vegetative growth, the *wt UBA2* was replaced by an otherwise *wt UBA2* that was marked with the *URA3* gene and expressed from the galactose-inducible, glucose-repressible P_{GAL1} promoter. The resulting heterozygous (P_{GAL1}-*UBA2*/*UBA2*) diploid was sporulated, and tetrads were dissected onto galactose-containing medium. The Ura^+ spores (in which *UBA2* was linked to P_{GAL1}) gave rise to smaller colonies than the Ura^- (*wt UBA2*) spores). However, no significant difference in colony sizes could be observed upon restreaking of the colonies (data not shown), suggesting that smaller sizes of the initial Ura^+ colonies were due to a phenotypic lag in the galactose-induced expression of *UBA2*. When P_{GAL1}-*UBA2* cells (strain JD62-6A) were streaked onto glucose-containing media, no single colonies were observed (Fig. 3), except for an occasional appearance of spontaneous suppressors (data not shown), indicating that *UBA2* is essential for vegetative growth as well. Microscopic examination of P_{GAL1}-*UBA2* cells that were grown in glucose for 15 h showed them to be arrested at more than one stage of the cell cycle. Many (not all) of these cells were larger than *wt* cells, and some of them had two or three buds (data not shown).

An Essential Cysteine in Uba2p—Uba2p contains the consensus sequence of *E*1 enzymes, K*XX*PZCT*XXXX*P (Z is a non-polar residue), which encompasses the essential Cys residue (C) that becomes linked to Ub in an *E*1~Ub thioester (see above and Fig. 2). To determine whether the corresponding Cys-177 of Uba2p (Fig. 2) is also essential for the function of Uba2p, we converted Cys-177 into Ala or Ser (see "Materials and Methods"). Low copy (*CEN*-based) plasmids expressing no Uba2p (vector alone), *wt* Uba2p, or its mutant variants Uba2p-C177A and Uba2p-C177S were introduced into strain JD62-6A, in which *wt UBA2* was expressed from P_{GAL1}, and the transformants were selected on galactose-containing (SG) plates. Upon restreaking, the transformants of all four classes yielded colonies of similar sizes on SG plates. However, only the transformants that carried the plasmid-borne *wt UBA2* grew on glucose-containing (SD) plates, conditions that repressed the expression of the chromosomal *UBA2* from P_{GAL1} (Fig. 3*A*). Thus, Cys-177 is required for an essential function of Uba2p.

We also determined the growth rate effects of overproducing either *wt* Uba2p or its inactive variants Uba2p-C177A and Uba2p-C177S. These proteins were expressed from high copy (2μ-based) plasmids and P_{GAL1} (Fig. 3*B*). Overproduction of *wt* Uba2p did not result in a significant growth defect (Fig. 3*B*). By contrast, the expression of either Uba2p-C177A or Uba2p-C177S in the same $Uba2^+$ cells greatly impaired their growth (Fig. 3*B*). Thus, *Uba2-C177A* and *Uba2-C177S* are dominant negative alleles of *UBA2*, suggesting that the corresponding inactive proteins (Uba2p-C177A and Uba2p-C177S) compete with *wt* Uba2p for binding to itself or other relevant proteins. This interpretation is consistent with the finding that Uba2p is a part of a heterooligomeric complex (see below).

Uba2p Is a Nuclear Protein—Uba2p was tagged at its C terminus with two tandem repeats of the 9-residue "ha" epitope (Field *et al.*, 1988), with the 8-residue "FLAG" epitope (Brizzard *et al.*, 1994), or with the 27-kDa green-fluorescent protein (GFP) from *Aequorea victoria* (Chalfie *et al.*, 1994), yielding, respectively, Uba2p-ha, Uba2p-FLAG, and Uba2p-GFP (see "Materials and Methods"). The GFP protein absorbs blue light and emits green light; it can be used as a fluorescent reporter in living cells (Chalfie *et al.*, 1994). The tagged derivatives of Uba2p were expressed from high copy plasmids and the copper-inducible P_{CUP1} promoter. The C-terminal extensions of Uba2p did not interfere significantly with its function: Uba2p-ha, Uba2p-FLAG, and Uba2p-GFP fully complemented the growth deficiency of the P_{GAL1}-*UBA2* strain JD62-6A on glucose-containing (SD) plates even in the absence of addi-

Fig. 1. **The *UBA2* gene of *S. cerevisiae*.** *Panel A*, restriction map of a segment of Chromosome IV encompassing *SPT3*, *SUF3*, *UBA2*, and *SAC7*. The location of *SPT3* (Winston and Minehart, 1986), *SUF3* (Mendenhall and Culbertson, 1988), and *SAC7* (Dunn and Shortle, 1990) was deduced from analyses of restriction fragments and determination of nucleotide sequences in the vicinity of *UBA2*. The complete sequence of the region between *SPT3* and *SUF3*, and between *UBA2* and *SAC7* is not yet available, leaving open the possibility of additional genes in these regions. The nucleotide sequence of *UBA2* has been submitted to the GenBank/EMBL data base with the accession number Z48725. *Dashed lines* indicate the region of *UBA2* that was replaced with *HIS3* in the *uba2*Δ allele (see "Materials and Methods"). *Panel B*, tetrad analysis of the *S. cerevisiae* strain JD90 (*uba2*Δ*::HIS3*/*UBA2*), a derivative of JD51 (see "Materials and Methods"). Tetrads were dissected onto YPD medium. The plate was photographed after 3 days incubation at 30 °C. All spores that gave rise to colonies were His^- (data not shown). *Panel C*, nucleotide sequence of the *UBA2* gene and deduced amino acid sequence of the Uba2p protein. Nucleotide and amino acid residues are numbered on the *left* and *right*, respectively. The sequence of a glycine $tRNA_{CCC}$ (the *SUF3* gene upstream of the *UBA2* ORF) is indicated by *shading*.

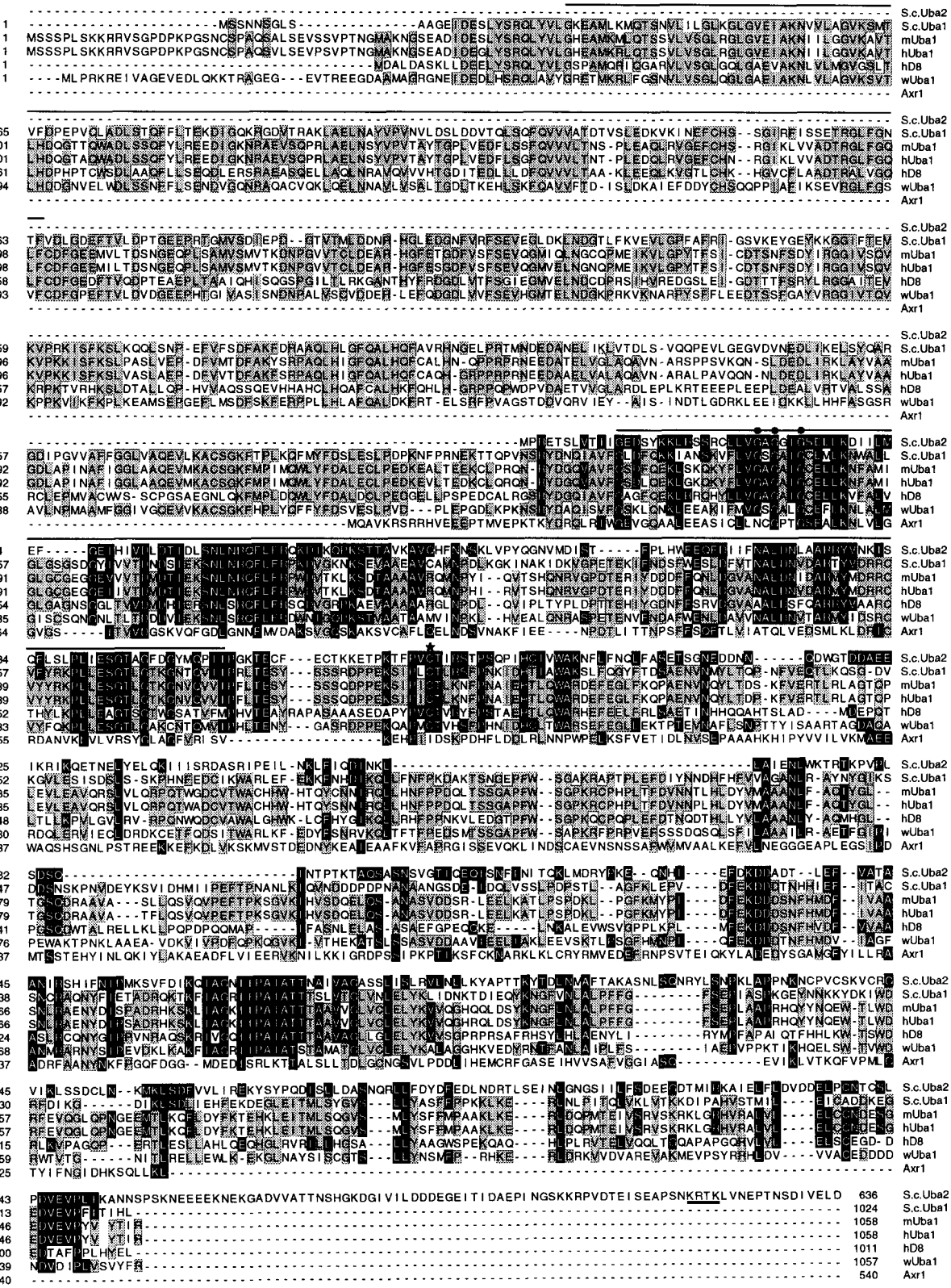

FIG. 2. **Sequence comparisons of Uba2p with *E*1 enzymes and the plant Axr1p protein.** Shown are the deduced sequences (in single-letter amino acid abbreviations) of the *S. cerevisiae* Uba2p (denoted as *S.c.Uba2*); the *S. cerevisiae* *E*1 enzyme Uba1p (denoted as *S.c.Uba1*) (McGrath *et al.*, 1991); the mouse *E*1 Uba1p (denoted as mUba1) (Imai *et al.*, 1992); human *E*1 (denoted as hUba1) (Handley *et al.*, 1991); human *E*1 homolog D8 (denoted as *hD8*), which is encoded by a gene in the chromosomal region 3p21 (Kok *et al.*, 1993); wheat *E*1 (denoted as *wUba1*) (Hatfield *et al.*, 1990); and the *A. thaliana* Axr1p protein (denoted as *Axr1*) (Leyser *et al.*, 1993). The sequences were aligned using the PileUp program (GCG package, version 7.2, Genetics Computer Group, Madison, WI). Gaps (indicated by *hyphens*) were used to maximize alignments. Residues identical between the Uba2p protein and at least one of the other proteins are *shaded* in *black*. Residues identical among at least three of the proteins other than Uba2p are *shaded* in *gray*. Residue numbers are on the *left* and also at the *end* of sequences. The position of a putative ATP-binding region (G*X*G*X*XG) is indicated by *black dots above* the sequence of Uba2p. The position of the putative active-site Cys-177 in Uba2p is marked by an *asterisk*. A putative NLS near the C terminus of Uba2p is *underlined*. Stretches that are partially duplicated within some of the proteins (Kok *et al.*, 1993) are indicated by *lines above* the sequence of Uba2p.

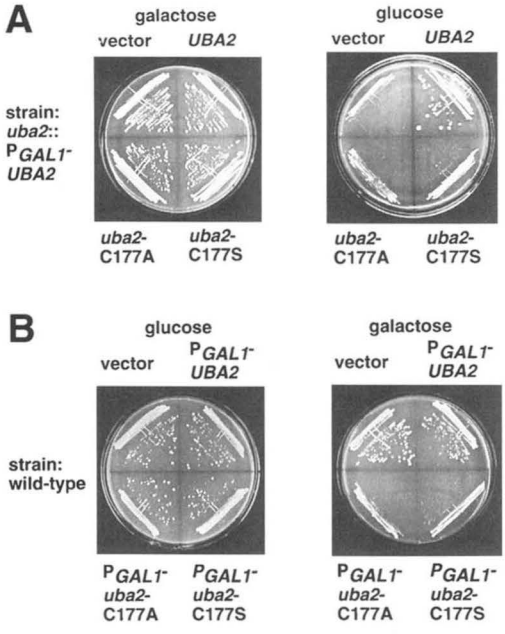

FIG. 3. **Cysteine 177 of Uba2p is essential for Uba2p function.** *Panel A*, the *S. cerevisiae* strain JD62-6A, in which *UBA2* was expressed from P_{GAL1} (see "Materials and Methods") was transformed with the *CEN6/ARSH4/LEU2* vector pRS315 (Sikorski and Hieter, 1989); or with the same vector expressing *wt* Uba2p; or with the same vector expressing either Uba2p-C177A or Uba2p-C177S. Transformants were selected on SG plates lacking uracil and leucine (SG/-Ura/-Leu), and were then restreaked onto the same medium (denoted as "galactose") or onto SD/-Ura/-Leu plates (denoted as "glucose"). *Panel B*, dominant negative effects of mutants at position 177 of Uba2p. The *wt* strain JD52 (a haploid segregant of JD51; see "Materials and Methods," and Fig. 1) was transformed with the high copy *LEU2* vector YEplac181 (Gietz and Sugino, 1988); or the same vector expressing (from P_{GAL1}) either *wt* Uba2p, or Uba2p-C177A, or Uba2p-C177S. Transformants were selected on SD/-Leu media, and were then restreaked onto the same (SD/-Leu) or SG/-Leu plates. The plates were photographed after 2 and 3 days of growth at 30 °C on glucose and galactose, respectively.

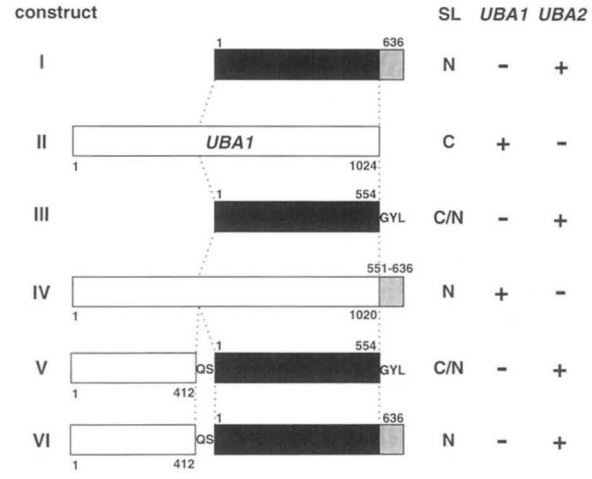

FIG. 4. **Uba1p and Uba2p have distinct and apparently non-overlapping functions.** Shown is an alignment of Uba1p and Uba2p derived from data in Fig. 2, and variants of these proteins that have been constructed and tested in the present work. The corresponding genes were expressed from P_{CUP1} in the high copy plasmid YEplac181 (see Fig. 3). The constructs were transformed into JD62-6A (P_{GAL1}-*UBA2*; see the legend to Fig. 3) or JD77-1A (*uba1Δ::HIS3*) carrying the low copy plasmid pJD320 that expressed *wt* Uba1p from P_{GAL1}. Transformants were selected on galactose-containing plates and then assayed for growth on glucose-containing plates as shown in Fig. 3. "+" on the right indicates colony growth on glucose-containing plates; "−" denotes the absence of a significant difference in the kinetics of growth arrest in comparison to that observed with the same strain carrying vector alone. Subcellular localization of these proteins was analyzed by fluorescent microscopy (Fig. 5), using fusions between the GFP and C termini of the above protein constructs (see "Materials and Methods"). These GFP fusions (whose properties in complementation assays were indistinguishable from those of their counterparts lacking the GFP moiety) were expressed in *wt S. cerevisiae* as described above for the GFP-lacking fusions. Subcellular localization (denoted as *SL*) of the GFP fusions is abbreviated in the diagram as "*C*" for largely cytoplasmic (apparently cytosolic) and "*N*" for largely nuclear (see Fig. 5). Uba2p-specific portions of these fusions are shaded in *black* or *gray*. Uba1p is represented as a *white rectangle*. Additional amino acid sequences at the C termini of truncated variants or at the junctions within specific fusion proteins are shown in single letter abbreviations. The positions of regions of identical or similar sequences among these constructs are indicated by *dotted lines*.

tional, P_{CUP1}-inducing levels of Cu^{2+} in the medium (data not shown).

Immunoblot analysis of extracts (prepared using a detergent-lacking lysis buffer) from cells expressing Uba2p-ha indicated that the bulk of Uba2p-ha was present in the 14,000 × *g* pellet, from which it could be recovered after resuspension of the pellet in a lysis buffer containing 1% Triton X-100 (data not shown), suggesting that Uba2p-ha was located in a membrane-enclosed compartment. Fluorescence microscopy was then used to examine *S. cerevisiae* transformants that expressed the Uba2p-GFP fusion protein. The bulk of fluorescent GFP was located in an organelle identified as the nucleus since it could also be stained with DAPI, a DNA-binding fluorescent marker (Fig. 5, *B* and *C*). In a control transformant that expressed GFP alone the bulk of fluorescent GFP was located in the cytoplasm and probably also in the nucleus, because no "negative" staining of the nucleus was observed (Fig. 5*I*). Fractionation experiments with extracts from *S. cerevisiae* that expressed Uba2-FLAG confirmed the nuclear localization of Uba2p (data not shown).

Uba1p and Uba2p Cannot Substitute for Each Other—We asked whether increased expression of Uba1p (the yeast *E*1 enzyme) could compensate for the absence of Uba2p, or vice versa. Uba1p was overproduced by expressing it from a high copy plasmid and the induced P_{CUP1} (see "Materials and Methods"). Overproduction of Uba1p in the P_{GAL1}-*UBA2* strain JD62-6A did not rescue these cells from growth arrest on glucose-containing media (conditions that repressed the expression of Uba2p). In a reciprocal test, Uba2p was expressed from a high copy plasmid and P_{CUP1} in a *uba1Δ* strain (McGrath *et al.*, 1991) that expressed Uba1p from a low copy plasmid and P_{GAL1} (see "Materials and Methods"). Overproduction of Uba2p did not prevent growth arrest of P_{GAL1}-*UBA1* cells on glucose-containing media (Fig. 4 and data not shown). A derivative of Uba2p that lacked its 82-residue C-terminal region (which is absent from the known *E*1 enzymes) (Fig. 4*III*) was also unable to rescue cells depleted for Uba1p. The same results were obtained with a Uba2p derivative bearing the 412-residue N-terminal region of Uba1p (this region is absent from Uba2p) (Fig. 4, *V* and *VI*). (Either of these Uba2p-based constructs could rescue cells depleted for Uba2p (data not shown).)

Fluorescence patterns of cells expressing Uba1p-GFP (this fusion was functionally active in that it could rescue *uba1Δ* cells) were indistinguishable from those expressing GFP alone (see above and Fig. 5*D*), suggesting that Uba1p was located in both the cytosol and the nucleus. Fluorescence patterns of cells expressing a GFP fusion to the Uba2p derivative bearing the *E*1-specific N-terminal region (Fig. 4*VI*) indicated that the presence of this region did not impair the accumulation of Uba2p in the nucleus (Fig. 5*H*). By contrast, a Uba2p-GFP fusion lacking the 82-residue C-terminal region of Uba2p (this region contained a putative NLS) (Fig. 4*III*) accumulated in the nucleus much less efficiently: the relative level of cytoplasmic fluorescence was much higher with this fusion than with GFP fusions to either *wt* Uba2p or Uba2p bearing the *E*1-specific

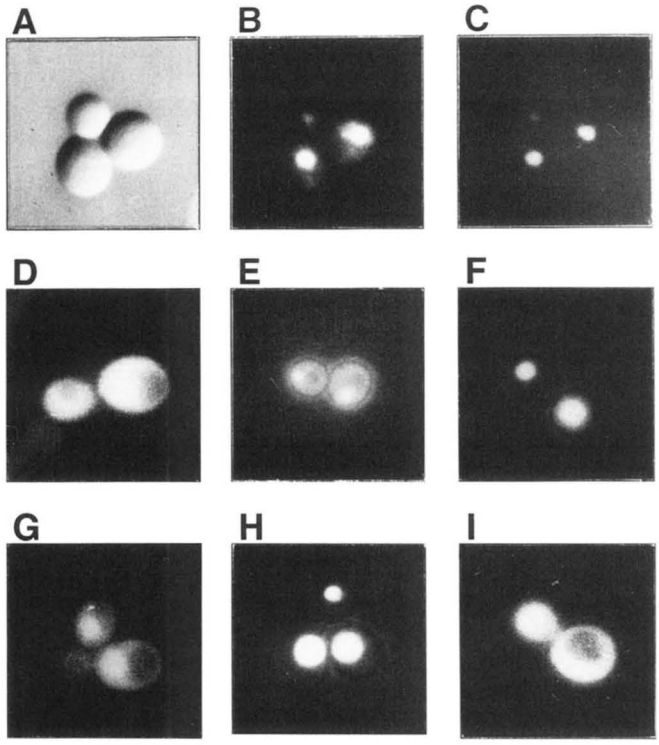

FIG. 5. **Uba2p is a nuclear protein.** The subcellular localization of Uba2p and related proteins diagrammed in Fig. 4 was analyzed using fluorescence microscopy of yeast transformants expressing these proteins as fusions to GFP (see "Materials and Methods"). *A*, cells expressing Uba2p-GFP (Fig. 4*I*), viewed with Nomarski optics; *B*, same as *A* but stained with DAPI; *C*, same as *A* but GFP-specific green fluorescence; *D-H*, GFP-specific green fluorescence of cells expressing the GFP-containing counterparts of the following constructs in Fig. 4: *II* (*D*), *III* (*E*), *IV* (*F*), *V* (*G*), and *VI* (*H*). *I*, cells expressing GFP alone. No green fluorescence was observed with cells lacking GFP (data not shown).

N-terminal region (Fig. 5*E*). Thus, the C-terminal region of Uba2p is important for its transport to the nucleus but may be not the sole NLS-containing region of Uba2p. That the C-terminal region of Uba2p actually bears a portable NLS was confirmed by fusing this region to the C terminus of Uba1p (Fig. 4*IV*). The resulting fusion was unable to rescue cells depleted for Uba2p (see above), but did rescue cells depleted for Uba1p (Fig. 4*IV*). Examination of cells expressing the corresponding GFP fusion (Fig. 4*IV*) showed that the bulk of the Uba1p derivative bearing the 86-residue C-terminal region of Uba2p was located in the nucleus (Fig. 5*F*). We conclude that the *S. cerevisiae* Uba1p (*E*1 enzyme) and Uba2p have distinct, apparently nonoverlapping, essential functions.

Does Uba2p Activate Ubiquitin?—The sequence similarities between Uba2p and the known *E*1 enzymes, and the functional essentiality of the conserved Cys-177 of Uba2p were consistent with the possibility that Uba2p might be a nuclear ubiquitin-activating (*E*1) enzyme. We therefore tested whether Uba2p could activate Ub *in vitro* under conditions permissive for Ub activation by Uba1p, the known *S. cerevisiae E*1 enzyme. Uba1p-FLAG and Uba2p-FLAG were precipitated with a monoclonal anti-FLAG antibody linked to an agarose-based affinity resin. The antibody-immobilized Uba1p-FLAG and Uba2p-FLAG were incubated in the presence of ATP and [^{35}S]methionine-labeled His6-Ub (see "Materials and Methods"). The incubation products were fractionated by SDS-PAGE with or without prior treatment with mercaptoethanol at 100 °C (this treatment cleaves Ub thioesters but not isopeptide bond-mediated Ub conjugates). While Uba1p-FLAG formed a Ub adduct (Uba1p~Ub thioester) sensitive to mercaptoethanol, no such adduct could be detected with Uba2p-FLAG (data not shown).

In a different test, we prepared an extract from a yeast strain that expressed both Uba1p-FLAG and His6-Ub, and used chromatography on Ni-NTA-Sepharose to retain His6-Ub and its covalent or noncovalent complexes with other proteins. The preparation of extract and its loading onto the column were carried out in the presence of ATP. Under these conditions, a fraction of Uba1p-FLAG in the extract bound to the column, and could be specifically eluted with a buffer containing AMP and pyrophosphate (see "Materials and Methods"). The binding of Uba1p-FLAG to Ni-NTA-Sepharose under these conditions resulted from the formation of a thioester between Uba1p-FLAG (an *E*1 enzyme) and the column-bound His6-Ub. Since AMP and pyrophosphate are the other products of this ATP-dependent reaction (Haas *et al.*, 1982), high concentrations of AMP and pyrophosphate can partially reverse it (Ciechanover *et al.*, 1982), yielding ATP and free *E*1 (Uba1p-FLAG).

In an otherwise identical test with cells expressing His6-Ub and Uba2p-FLAG (instead of Uba1p-FLAG), no binding of Uba2p-FLAG to the column containing His6-Ub was observed (data not shown). We conclude that Uba2p does not form a thioester with Ub under conditions that allow the formation of Ub thioesters by a known *E*1 enzyme such as Uba1p.

Uba2p Is Associated with Itself and Other Proteins—To examine the interactions of Uba2p with other *S. cerevisiae* proteins, we expressed various Uba2p derivatives tagged with the ha epitope in the strain JD62-6A (which expressed *wt* Uba2p from P_{GAL1}). Cells expressing Uba2p-ha, Uba2p-C177A-ha, Uba2p-C177S-ha, or Uba2p-GFP-ha were grown in SG medium; a portion of this culture was shifted to the glucose-containing SD medium for 8 h before labeling with [^{35}S]methionine (these conditions extinguished the expression of *wt* Uba2p from P_{GAL1}). SDS-PAGE of proteins immunoprecipitated from whole cell extracts with anti-ha antibody yielded not only the expected Uba2p species but several other proteins as well (Fig. 6). Uba2p-ha (and its Cys-177 derivatives) ran as doublets with apparent molecular masses of ~88 and ~90 kDa, significantly higher than the calculated molecular mass of Uba2p-ha (73 kDa). The same anomalous mobility was observed with Uba2p expressed in *E. coli* (data not shown). The ~90-kDa form was also detected with anti-FLAG antibody in immunoblot analyses of cells expressing Uba2p-FLAG (Fig. 7). The ~90-kDa Uba2p appears to be a phosphorylated derivative of the ~88-kDa Uba2p.[4]

Several non-Uba2p species (termed p52, p63, p87, and p120), with apparent molecular masses of ~52, ~63, ~87, and ~120 kDa, were specifically coimmunoprecipitated with Uba2p-ha (Fig. 6). The same proteins were coimmunoprecipitated with either the functionally active Uba2p-ha (Fig. 6, *lanes 2, 5*, and *6*) or the inactive (mutant at position 177) derivatives of Uba2p-ha (*lanes 3* and *4*), indicating that Uba2p interactions with these proteins do not require its putative active-site Cys-177. (Proteins of the same molecular masses were also copurified together with Uba2p-FLAG-His6 fusion on either an anti-FLAG antibody (agarose-based) affinity resin or on Ni-NTA-Sepharose; these results (data not shown) provided independent evidence for a direct interaction between Uba2p and at least one polypeptide among p52, p63, p87, and p120.) The electrophoretic mobilities of Uba2p-interacting proteins were the same irrespective of whether the samples were treated with mercaptoethanol before SDS-PAGE (data not shown).

Immunoprecipitation with anti-ha antibody was also carried

[4] R. J. Dohmen, unpublished data.

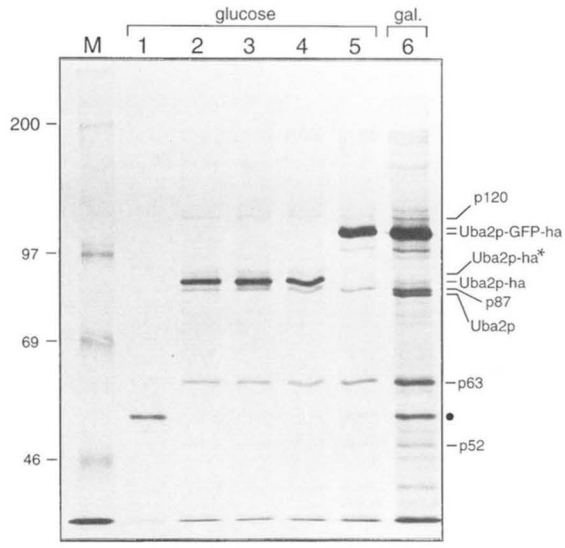

FIG. 6. **Uba2p interacts with itself and other proteins.** ^{35}S-Labeled proteins from the strain JD62-6A (P_{GAL1}-UBA2; see the legend to Fig. 3) transformed with either a vector (control) or vector-based plasmids expressing ha-tagged Uba2p derivatives were immunoprecipitated with a monoclonal anti-ha antibody, and analyzed by SDS-PAGE and fluorography. Each of the samples was split in two and either heat-treated in the presence of mercaptoethanol before SDS-8% PAGE (see "Materials and Methods") or left untreated. The data shown here were obtained with untreated samples (mercaptoethanol-treated samples yielded the same results). The cells carried either a control vector (*lane 1*) or plasmids expressing Uba2p-ha (*lane 2*), Uba2p-C177A-ha (*lane 3*), Uba2p-C177S-ha (*lane 4*), or Uba2p-GFP-ha (*lanes 5* and *6*). The cells were grown in galactose and shifted to a glucose-containing medium 8 h before ^{35}S labeling (*lanes 1–5*). Alternatively, the cells were labeled in a galactose-containing medium (*lane 6*). The sizes of marker proteins (M) are in kDa, on the *left*. Positions of different Uba2p derivatives and coimmunoprecipitated proteins (denoted as p52, p63, p87, and p120) are indicated on the *right*. *Uba2p-ha** denotes a post-translationally modified Uba2p-ha (see also Fig. 7 and main text). A *dot* marks the position of a *S. cerevisiae* protein that cross-reacted with anti-ha antibody (Dohmen *et al.*, 1991a).

out with an otherwise identical extract prepared from JD62-6A cells grown in the presence of galactose; these cells, unlike those grown on glucose-containing media, contained the untagged *wt* Uba2p as well. This immunoprecipitation yielded, in addition to the non-Uba2p species described above, a protein with an apparent molecular mass (~86 kDa) expected for the untagged, *wt* Uba2p (Fig. 6, *lane 6*). (The expected apparent mass of the untagged Uba2p was calculated by subtracting 2 kDa (the mass of ha tag) from 88 kDa, the apparent mass of the 73-kDa Uba2p-ha.) In addition, extracts from cells lacking *wt* Uba2p and expressing both Uba2p-FLAG and Uba2p-GFP-ha yielded both Uba2p-FLAG and Uba2p-GFP-ha upon immunoprecipitation with either anti-FLAG or anti-ha antibody (data not shown). Taken together, these findings (Fig. 6 and data not shown) strongly suggested that Uba2p interacts with itself and is a part of a complex or complexes that contain proteins p52, p63, p87, and p120.

Uba2p Is Ubiquitinated—In the course of testing for the putative Ub-activating function of Uba2p, we found that Uba2p-FLAG is multiubiquitinated *in vivo* (Fig. 7). In these experiments, the anti-FLAG antibody was used to immunoprecipitate proteins from extracts of the *S. cerevisiae* strain SUB312, which lacked the chromosomal Ub-coding sequences, expressed either *wt* Ub or epitope-tagged mycUb from a plasmid (Finley *et al.*, 1994; Ellison and Hochstrasser, 1991), and in addition carried either a plasmid expressing Uba2p-FLAG (from the induced P_{CUP1}) or a corresponding control vector (see "Materials and Methods"). Immunoblot analysis, using a monoclonal anti-myc antibody, of proteins that had been immunoprecipitated with anti-FLAG antibody from cells expressing both Uba2p-FLAG and mycUb showed an extensive smear of mycUb-containing species extending from below the position of Uba2p-FLAG to the top of the gel (Fig. 7*B*). This pattern was observed exclusively with cells expressing both Uba2p-FLAG and mycUb, and was the same irrespective of whether the samples were heat-treated in the presence of mercaptoethanol or left untreated before SDS-PAGE; the latter result indicated that most species in the mycUb-containing smear lacked mycUb thioesters (Fig. 7 and data not shown).

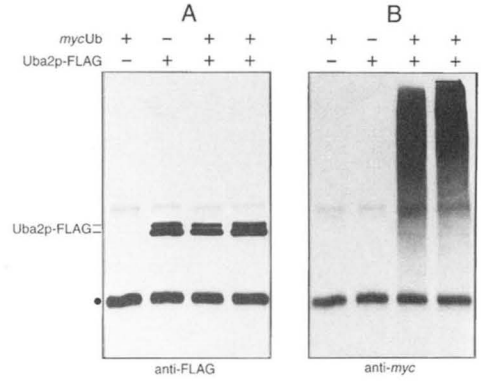

FIG. 7. **Post-translational modifications of Uba2p.** Immunoblot analyses of proteins immunoprecipitated with the monoclonal anti-FLAG antibody from the *S. cerevisiae* strain SUB312 (Finley *et al.*, 1994) that lacked the chromosomal Ub-coding sequences, expressed either *wt* Ub or mycUb from a plasmid, and in addition carried either a plasmid expressing Uba2p-FLAG or a corresponding (control) vector (see "Materials and Methods," and the main text). *Panel A*, immunoblotting with anti-FLAG antibody after SDS-8% PAGE of proteins immunoprecipitated with the same antibody. The presence (or absence) of mycUb and Uba2p-FLAG in each of the initial extracts is indicated above the lanes. An extract used to produce the sample in the *third lane* was identical to an extract in the *fourth lane* except that the latter was heated at 100 °C in the presence of 0.6% SDS before immunoprecipitation with anti-FLAG antibody (see main text). *Panel B*, the immunoblot in *A* was reprobed with a monoclonal anti-myc antibody. The position of a heavy chain of anti-FLAG antibody that reacted with the secondary antibody is indicated by a *dot* on the *left*.

No smear of mycUb-containing species was observed in otherwise identical experiments with cells that expressed mycUb and Uba1p-FLAG (but not Uba2p-FLAG). Instead, a band corresponding to the expected size of a Uba1p ~ mycUb thioester was detected by the anti-myc antibody; this band was not observed with mercaptoethanol-pretreated samples (data not shown). Since no smear of Uba2p-FLAG-containing species was detected in the samples immunoprecipitated with anti-FLAG antibody and then immunoblotted with the same antibody, we asked whether mycUb moieties were linked directly to Uba2p-FLAG or to a protein(s) coimmunoprecipitated with it by anti-FLAG antibody. The lysis buffer (see "Materials and Methods") was made 0.6% in SDS; the extract was heated at 100 °C for 3 min, and then diluted with SDS-lacking lysis buffer to the final SDS concentration of 0.03%, followed by immunoprecipitation with anti-FLAG antibody. The results were indistinguishable from those obtained in the absence of SDS pretreatment (Fig. 7), strongly suggesting that the mycUb-containing species of the smear were largely those of multiubiquitinated Uba2p-FLAG.

This interpretation was supported by the results of experiments in which Uba2p-FLAG-His6 was expressed in cells that also expressed mycUb. Uba2p-FLAG-His6 was purified from an extract of these cells on Ni-NTA-Sepharose in the presence of 8 M urea, conditions expected to disrupt noncovalent protein-protein interactions but not the isopeptide bonds of Ub conjugates. Immunoblotting with anti-myc antibody still detected mycUb in fractions from the column that contained Uba2p-FLAG-His6 (data not shown).

Taken together, these findings (Fig. 7 and data not shown) suggest that a relatively small proportion of Uba2p-FLAG is ubiquitinated *in vivo*, because otherwise these multiubiquitinated Uba2p-FLAG species should have also been detectable with anti-FLAG antibody. (The sensitivity of detection of mycUb-containing multiubiquitinated Uba2p-FLAG species would be expected to be higher with anti-myc than with anti-FLAG antibody, given the increasing molar ratio of mycUb to Uba2p-FLAG in higher molecular mass species of the smear.) The observation that a minor fraction of the smear of mycUb-containing species extends below the position of Uba2p-FLAG (Fig. 7*B*) suggests, among other possibilities, that multiply ubiquitinated Uba2p-FLAG is proteolyzed *in vivo*.

Concluding Remarks—We describe the isolation and analysis of an essential *S. cerevisiae* gene, termed *UBA2*, that encodes a 71-kDa nuclear protein Uba2p with extensive sequence similarities to Ub-activating (*E*1) enzymes, an evolutionarily conserved family of 110–120-kDa proteins. Until the finding of Uba2p, the previously identified *S. cerevisiae* Uba1p *E*1 enzyme (McGrath *et al.*, 1991) has been the only known member of the *E*1 sequence family in this organism.

A variety of tests for E1 activity of Uba2p, the ability to form a thioester with Ub, yielded negative results (see above), suggesting that Uba2p is not an *E*1 enzyme. We also showed that Uba2p, which is located largely in the nucleus, cannot complement the essential function of the largely cytosolic Uba1p *E*1 enzyme even if Uba2p is rendered partially cytosolic through a deletion of its NLS-containing C-terminal region. Conversely, Uba1p cannot complement the (unknown) essential function of Uba2p even if Uba1p is made partially nuclear by fusing it to the NLS-containing C-terminal region of Uba2p.

One constraint on the range of possible functions of Uba2p was provided by the finding that Cys-177 of Uba2p is required for an essential (cell viability-sustaining) function of Uba2p. Cys-177 of Uba2p is located within a region conserved among *E*1 enzymes and at a position where the active-site cysteine is present in the known *E*1s. (This cysteine forms a thioester bond to the C terminus of Ub in an *E*1~Ub thioester (see Introduction).) Thus it is likely that an enzymatic reaction catalyzed by Uba2p involves the formation of a Cys-177-mediated thioester between Uba2p and either Ub or another protein. One possibility is that Uba2p acts as a Ub-conjugating (*E*2) enzyme (see Introduction). If so, Uba2p would be the first example of an *E*2 whose sequence is *E*1-like and dissimilar to sequences of the currently known *E*2 enzymes.

Another clue that should help understand the function of Uba2p was provided by the finding that Uba2p interacts with itself and several other, presently unknown proteins, termed p52, p63, p87, and p120 (Fig. 6). Uba2p was also found to be multiubiquitinated *in vivo* (Fig. 7), suggesting that at least a fraction of Uba2p is metabolically unstable.

We recently learned that C. Moore and her colleagues had also identified the *UBA2* gene, which they termed *PIP2* (for *p*olymerase-*i*nteracting *p*rotein 2).[5] Pip2p (Uba2p) was isolated in a two-hybrid screen (Chien *et al.*, 1991) for proteins that interact with Pap1p, the poly(A) polymerase of *S. cerevisiae*. *PAP1* is essential for cell viability and encodes a 64.5-kDa protein (Lingner *et al.*, 1991). Uba2p (Pip2p) is apparently not required for mRNA processing but plays a role in its regulation.[5] We note that p63, a protein of the size (~63 kDa) similar to that of Pap1p, coimmunoprecipitated with the Uba2p protein in our experiments (Fig. 6). Pap1p is also known to interact with CF1, a cleavage and polyadenylation specificity factor whose components include Rna14p and Rna15p (Chen and Moore, 1992; Minvielle-Sebastia, 1994). The presence of active Uba2p in these or other heterooligomeric complexes appears to be essential for cell viability, because overexpression of inactive Uba2p derivatives that lack the putative active-site Cys-177 residue has a dominant negative effect on cell growth (Fig. 3). It is likely that Uba2p (Pip2p) regulates the rate or specificity of mRNA polyadenylation by mediating ubiquitination and possibly also degradation of at least one component of a machinery involved. Further work, currently under way, is aimed at identifying this component and other aspects of the Uba2p function.

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[5] M. del Olmo, S. Gross, and C. Moore, manuscript in preparation.

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